Production of Botulinum Toxin A

BE 3340 Process Design Group 5

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Clostridium botulinum, although one of the most deadly toxin-producing bacteria known to man, also employs a vital purpose in medical drug therapy. Through the growth, extraction, and purification of Botulinum Toxin A (BT-A) from either live cultures or spores of *C. botulinum*, patients suffering from a multitude of neuromuscular disorders are given more freedom to live independently and painlessly. Specifically, in the neuromuscular disorder Cerebral Palsy, an excess release of acetylcholine results in uncontrolled muscle contraction. BT-A inhibits the transfer of acetylcholine in the neuromuscular junction and thereby prevents muscle contraction. Methods: Commonly used methods for BT-A production begin with growing and fermenting *C. botulinum* spores until reaching a specified concentration. The toxin itself is removed from the fermented beer through acid precipitation and centrifugation. Multiple types of chromatography can be used for further purification of the toxin, and the concentrated substance is fully polished through crystallization. The final product is diluted in a human serum medium as a liquid or freeze dried to store in the solid state. Objective: Our purpose is to develop an efficient process for mass producing BT-A for medicinal uses.

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Objective

The objective of this project is to design a process to isolate Botulinum neurotoxin A from a 50 L fermentation culture, dilute with human serum medium, and distribute into separate vials each containing 100 units (1 unit = 1 mouse LD50) of toxin for clinical use.

I. Introduction

a. Neurotoxins

Neurotoxins are one of the most overlooked causes of disease in society today. These multifaceted compounds generally swarm toward neurons in the body, preventing normal function by blocking various ion channels within the nervous system. While the human body naturally produces some neurotoxins that aid in vital processes, most harmful neurotoxins originate from other organisms. Reptiles, insects, amphibians, fungi and bacteria are some species that gain recognition for emission of neurotoxic substances. These creatures secrete toxins as a form of protection, making them poisonous when encountered by other organisms. Alternatively, neurotoxins occur naturally in the form of heavy metals. Both types of these toxins cause illnesses of various severities (Neurotoxin Basic Science).

The severity of the case depends on the type of toxin, the amount of the toxin, and the toxin's path of entry. The most commonly associated symptoms with neurotoxin contact are nausea, vomiting, diarrhea, fatigue, muscle cramps, and dizziness. These symptoms typically arise after ingestion of a neurotoxin and are the least severe side effects. More severe cases involve the inhalation of the toxin, which often leads to a quick limb paralysis and induction of a coma. In extreme cases, permanent nerve damage results. This nerve impairment leads to paralysis of both the peripheral and central nervous systems. Once the central nervous system becomes affected, respiratory paralysis ensues, eventually leading to death. Only a few toxins produced by organisms are potent enough to cause this rapid death in humans. Of these deadly poisons, the most common is the toxin belonging to the bacteria species *Clostridium botulinum* (Mergel).

b. Botulinum Toxin

Botulinum Toxin is a neurotoxin that is produced by the *Clostridium botulinum* bacterium ("Botulinum Toxin"). This common bacterium is infamous for causing Botulism, a serious, but rare food-borne illness that has paralytic effects as mentioned in detail above ("Botulism"). *Clostridium botulinum* spores are classified according to genotypic and phenotypic similarities into one of four groups, known as groups I-IV; however, these four groups can differentiate into seven different types of neurotoxins (A-G) based on their differing serological properties. Toxins A, B, or F can be produced from group I of *Clostridium botulinum*, and toxins B, E, or F can be produced from group II. Cases of Botulism in humans result from exposure to groups I or II, while group III causes Botulism only within plants. No onset of illness has been observed after exposure to group IV *C. botulinum* (Lindstrom). The Botulinum Toxins that create illness in humans block the release of the neurotransmitter, acetylcholine, which is described in detail below as the necessary component in muscle contraction (Nigam).

The structure and subunits of Botulinum toxin A (BT-A) enable it to prevent muscle function and contraction. Botulinum toxin A has a molecular weight of approximately 900 kDa ("Botulinum Toxin"). While the bulk of the toxin is made up of accessory proteins, the toxic portion of these neurotoxins comes from a single polypeptide chain composed of a 100 kDa heavy (H) chain and a 50 kDa light (L) chain. The light and heavy chains are connected by a single disulfide bond. In the absence of BT-A, an action potential across the motor neuron allows acetylcholine vesicles to be transported into the synaptic cleft (Lindstrom). Once vesicles are released into the synaptic cleft, they release the neurotransmitter acetylcholine and muscle contraction follows. When BT-A is present in the neuromuscular junction, the H-chain binds to the membrane of the acetylcholine vesicles in the presynaptic neuron and enters via endocytosis (Nigam). At this point, the disulfide bond between the H and L-chains is broken. The L-chain, still in the cytoplasm, moves to the SNARE complex at the presynaptic nerve terminal. The SNARE terminal is a group of proteins designed to enhance binding affinity of the acetylcholine vesicle to the nerve terminal. The L-chain's interactions with the SNARE complex disable the vesicles from binding, which prevents acetylcholine from being released into the synaptic cleft, and therefore, disables further muscle contraction (Nigam). Figure 1, shown below, depicts the neuromuscular junction in the absence and presence of BT-A. The right picture shows the BT-A after the disulfide bond breaks and before the light chain reaches the SNARE complex.

When a human contracts Botulism, the toxin spreads throughout the entire body, which leads to systemic paralysis, subsequent complications, and usually death. However, when Botulinum toxin A is highly purified into a pharmaceutical product, a dose directly injected into a muscle of interest allows for the toxin to be used as a therapeutic drug (Nigam). Unlike pathogenic Botulism that is ingested, BT-A is injected directly into the muscle of interest keeping the therapeutic toxin from spreading throughout the entire body. Patients suffering from neuromuscular diseases that limit muscular function are prime candidates for receiving this injection treatment. Botulinum toxin A is the only serotype currently available for these therapies and dosage amounts are tailored to each individual. BT-A has an onset of 1-3 days and a duration of action of 2-3 months (Nigam). Treatment of BT-A for patients with neuromuscular diseases relaxes the targeted muscles to increase comfort and functionality. Relaxation of the targeted muscles also allows physical therapy to improve function and range of motion of these areas (Bernard).

Since neuromuscular diseases include a multitude of disorders without a known cure, improving their quality of life is the ultimate goal. With the research found about the effectiveness of Botulinum Toxin A as a treatment for neuromuscular disorders, it greatly enhances the quality of life of patients by improving walking and range of motion, but most importantly it temporarily relieves the patient's pain from muscle tension. For these reasons, we decided to further research and enhance its production. It is a pharmaceutical product in great demand and has potential for improvement in its strength, specificity, and duration.

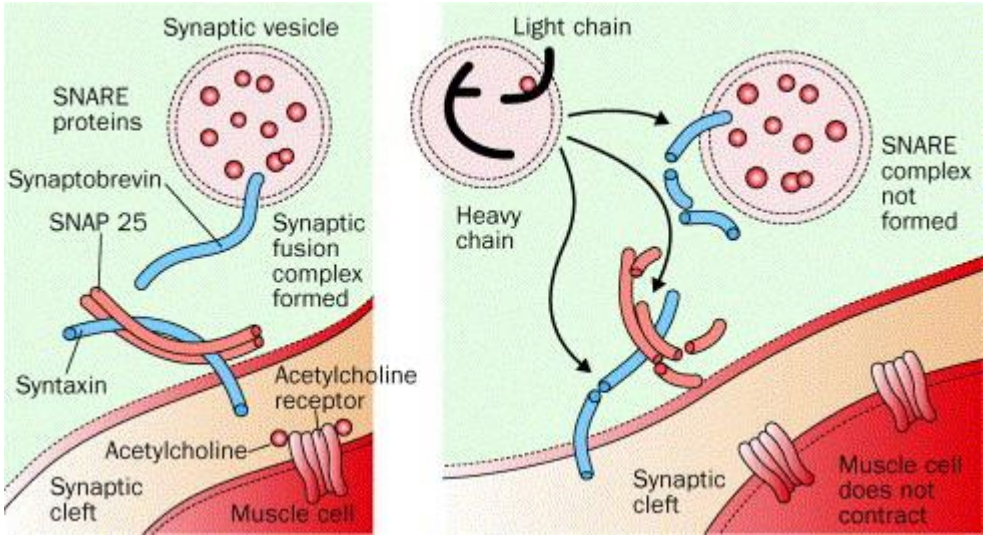


Figure 1: Normal neuromuscular junction (left); Neuromuscular junction in the presence of BT-A (right).
http://www.sciencedirect.com/science/article/pii/S0140673604162077

c. Applications of Botulinum Toxin

One of the specific diseases that benefits from Botulinum Toxin A treatment is Cerebral Palsy. Cerebral Palsy (CP) is a neuromuscular disease caused by a variety of possible damage to the central nervous system that develops during prenatal and early postnatal life. With the disease's early onset and short life expectancy, it is the most common cause of severe physical disability in children, affecting 1 in 400 children (Ubhi 2000). Their nervous system releases an excess amount of acetylcholine, causing continuous hypercontraction. It causes a multitude of motor dysfunctions such as spasticity, paresis, involuntary muscle contraction or dystonia, and loss of control of muscle predominantly in the upper or lower limbs. The patient, therefore, has poor posture, clenched hands with very little finger/thumb coordination, and trouble walking properly, all due to the long-lasting muscle contractions. For many years, treatment for Cerebral Palsy patients has involved physical therapy (PT), Occupational Therapy (OT), and orthotic braces. Although these forms of treatment have shown positive long-term progress, an intramuscular injection of Botulinum Toxin A has proven to be a much more effective treatment. It is currently the treatment administered to Cerebral Palsy patients in most clinics and hospitals nationwide.

Botulinum Toxin type A (BT-A) is used as treatment for involuntary muscle spasticity due to its mode of action causing denervation and muscle relaxation of a specific region. When the acetylcholine is inhibited, a Cerebral Palsy patient's muscle spasticity and tone is decreased significantly. For the past decade, clinical experience from an array of case reports and randomized controlled trials have shown the effectiveness of BT-A to treat spasticity in both upper and lower limbs in children with CP (Lukban et al. 2009). The FDA approved forms of BT-A that are currently on the market include Botox®, Dysport®, and Xeomin®, all produced from the US company Allergan. These have proven to reduce muscle tone and therefore improve functional outcome in countless Cerebral Palsy patients.

The motor function improvements of BT-A have been carried out through randomized, double blind, placebo-controlled trials. In the specific clinical trial run by Dr. Ubhi of the Academic Department of Pediatrics and Child Health in the UK, a group of 40 Cerebral Palsy patients were enrolled, 22 received BT-A, and 18 received a placebo as the control group. The effects were measured by video gait analysis (VGA), a digital software that records and quantifies specific posture and mechanics of motion. Results were further quantified by gross motor function measure (GMFM), physiological cost index (PCI), and passive ankle dorsiflexion. Video gait analysis showed significant improvements clinically and statistically six weeks and twelve weeks after BT-A treatment compared to the placebo (Ubhi 2000). One of the most common hindrances in cerebral palsy patients is in their initial foot contact in walking because the constantly contracted calf muscle prevents the normal heel to toe motion. In Dr. Ubhi's trial, video gait analysis showed clinical improvement in 48% of patients treated with BT-A in their initial foot contact, compared to only 17% improvement in the placebo treated patients. The gross motor function measure showed further statistical data in support of the treatment of Botulinum Toxin A. These results show how BT-A effectively relaxes the spastic muscles of targeted muscles, allowing improvement in gait and motor function of cerebral palsy conditions. In addition, trials have been completed with little to no known side effects. The only complaint of patients is soreness at the site of injection. With the proven effects of BT-A, more research and trials can be done to improve the drug's quality, specificity of muscles, and alteration of doses for long term relief.

II. Production Process

a. Raw Materials

The process of botulinum toxin A production begins with culturing *Clostridium botulinum* in proper conditions. This species of bacterium is bacillus shaped with a thick peptidoglycan layer, characteristic of Gram-positive bacteria. In addition, *C. botulinum* is an obligate anaerobe, meaning it will die in the presence of oxygen. Due to this, the bacterium colonizes in soils and in the intestinal tract of birds, fish, and some mammals. It is a very versatile organism and forms endospores when put in stressful environments (Parkinson). These spores are capable of withstanding very harsh living conditions, especially high temperatures, making them very difficult to kill. With the proper conditions, the spores proliferate and begin producing toxins (Infectious Disease Index).

To provide the proper nutrients, the medium chosen for growth usually has animal or soy product. These growth mediums must contain glucose or another similar carbohydrate to promote toxin production. Trypticase is also another main component in the mediums used, and can be substituted with tryptone. Some of the common growth mediums that contain these components include: tryptose sulfite cycloserine (TSC), trypticase-peptone-glucose-yeast extract broth (TPGY), and anaerobic egg yolk agar (Solomon). The following tables 1, 2, and three show the chemical component breakdown of TPGY broth, TSC broth, and anaerobic egg yolk agar.

Tryptose	833.33 g
Yeast extract	277.78 g
Soytone	277.78 g
Ferric ammonium citrate (NF Brown Pearls)	55.56 g
Sodium metabisulfite	55.56 g
Distilled water	50 liter

Table 1: Base Contents of TSC Broth (modified from *Solomon* to make 50 L)

Trypticase	2500 g
Peptone	250 g
Yeast extract	1000 g
Dextrose	200 g
Sodium thioglycollate	50 g
Distilled water	50 liter

Table 2: Base Contents of TPGY Broth (modified from *Solomon* to make 50 L)

Yeast extract	250 g
Tryptone	250 g
Proteose peptone	1000 g
NaCl	250 g
Agar	1000 g
Distilled water	50 liter

Table 3: Base Contents of Anaerobic Egg Yolk Agar (modified from *Solomon* to make 50 L)

TSC is the growth medium typically chosen to promote maximum growth of the organism. This medium contains all of the necessary amino acids that *C. botulinum* uses for metabolic growth and selects against other competitive microbes. With this broth and under ideal growth conditions, the most rapid doubling time possible is 30 minutes, allowing for quick and optimal toxin production (Solomon).

In addition to the correct medium, many other raw materials and equipment are used in the production of the botulinum toxin, and are listed below in table 4.

	Material	Characteristics
Biological Agents		
	C. botulinum	BSL-2
	Human serum albumin	From human plasma; 30% solution, protease free
Chemical Agents		
	TSC growth medium	
	Sodium metabisulfate	Allergen, carcinogen
	Tryptose	Nontoxic
	Ferric ammonium citrate	Irritant, toxic
	Yeast extract	Nontoxic
	Soytone	Nontoxic

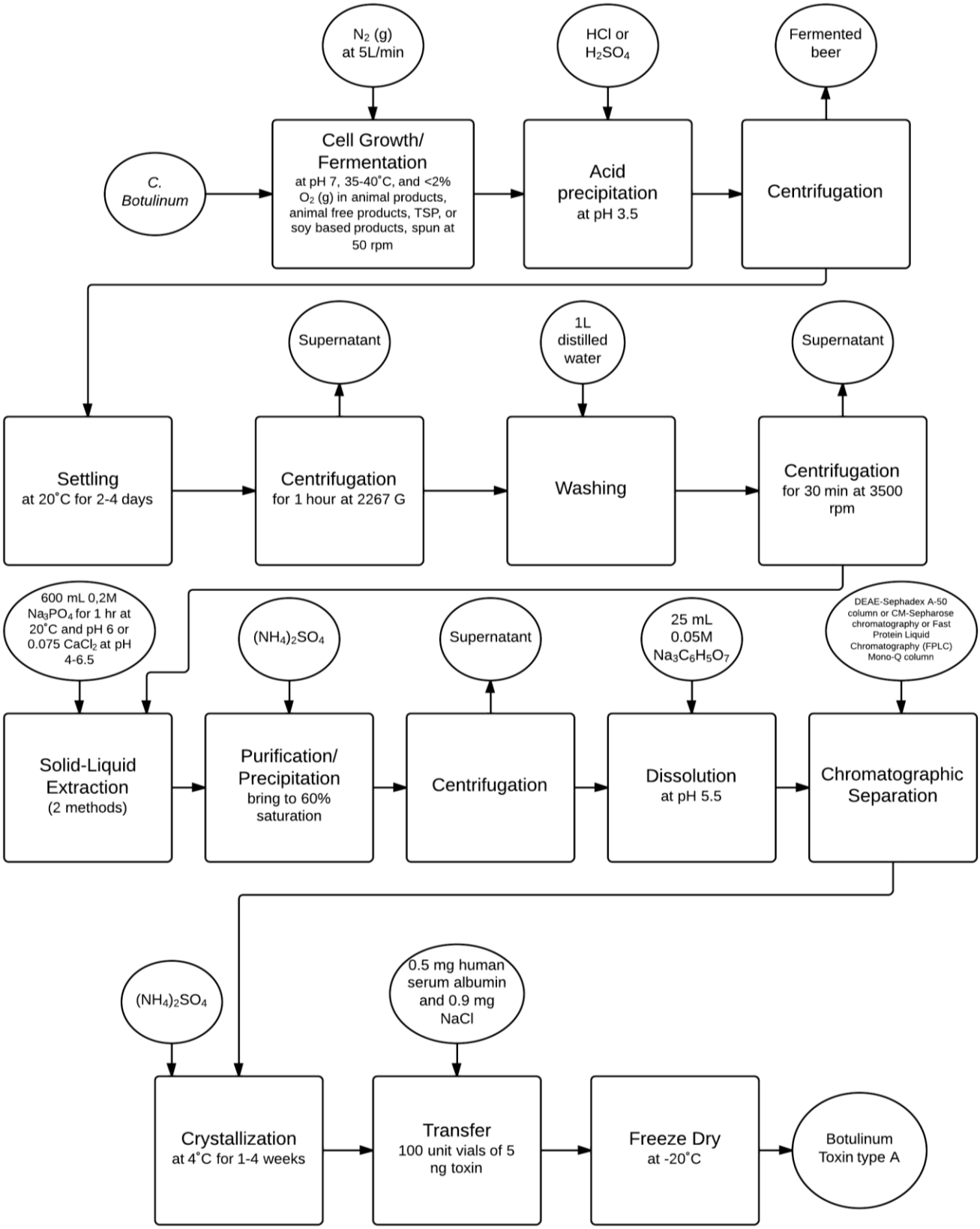
	Distilled water	Nontoxic
	Nitrogen gas	Nontoxic
	Hydrochloric or sulphuric acid	Irritant, corrosive
	Distilled water	Nontoxic
	Sodium phosphate buffer	Irritant, allergen
	Calcium chloride	Irritant
	Ammonium sulfate	Mild irritant
	Sodium citrate buffer	Mild irritant
	Sodium chloride	Nontoxic
Equipment		
	Incubator	Capable of 37°C
	Industrial fermenter	50 L capacity, sterilization, temperature, gas flow, and agitation control
	Vertical Decanter Centrifuge Model DRC 60	Capacity: 60 L; Centrifugal force: 2267 G
	DEAE-Sephadex A-50 column	Weak anion separator; max operating linear flow rate: 45 cm/hr at 25°C
	CM-Sepharose Chromatography Column	Bead diameter: 40-165 μm; 10- 4,000 kDA fractionation range
	Fast Protein Liquid Chromatography (FPLC) Mono-Q Column	50 x 5 mm, bead volume: 1mL; 0.2-2 mL/min flow rate; max loading capacity: 25 mg; protein binding capacity: 65 mg human serum albumin

Table 4: List of Raw Materials and Equipment with Characteristics
*all above information found at www.sigmaaldrich.com and www.msdsonline.com

b. Overview of Production Process

In order to be acceptable for clinical use as in the objective of this process, the toxin must be produced under strict conditions that guarantee quality, safety, and efficacy standards. The process begins with cultures of *Clostridium botulinum* type A grown from a seed stock, inoculated into a fermenter where it grows under anaerobic conditions. From here, the steps of the idealized process are not carried out in the ideal order due to the nature and properties of obtaining this product. Once the toxin reaches a maximum level of potency, the fermentation culture is acidified to precipitate the extracellular toxin from the broth. The crystalline precipitate is then separated by centrifugation for easy recovery. Traditionally, the purification process is then carried out through consecutive acid precipitations and centrifugation, followed by extractions through chromatography. Recent methods involve the original acidification and extraction procedures, but are followed by purifying the toxin with three High-Performance (HP) Chromatography steps (Simpson). These purification processes yield a bulk active substance containing the heavy and light chain toxin, and are stored in a freezer at or below -5°C. In preparing for clinical formulation, it is diluted in human serum albumin, and separated into vials. The vials are freeze-dried or vacuum-dried to protect the toxin and act as a bulking agent for the product (Hambleton 1992). 1 unit of toxin will be defined as 1 mouse lethal dose as determined by a lethal dose assay (~1.2 ng).

c. General Flowchart



d. Growth and Fermentation

BT-A is produced as a byproduct of the *C. botulinum* natural metabolism. Growth conditions must be carefully tuned to the bacteria's ideal growth conditions for an efficient process. Optimal toxin production occurs in a specific environment: bacterial growth is at its maximum, and the toxin itself is not damaged by the outside temperatures or pH. These desired conditions are near neutral 7 pH, a temperature between 35-40°C, and less than 2% oxygen present (Bonventre). Media may include animal products or soy-based products (Ton et Al). Tryptose Sulfite Cycloserine has been shown as an effective medium for growth (Solomon). *C. botulinum* will grow under these regulated conditions until the maximum toxin concentration is reached at 24 hours (Siegel).

A seed culture of *C. botulinum* type A is inoculated in a growth medium to increase the number of microbes available for fermentation. The seed culture can be obtained from spores or living cells. Spores are more robust than cells for long term storage, but involve additional processing steps to produce and activate the spores. Our process will use *C. botulinum* spores for growth because the costs associated with sporulation and activation can be considered negligible (Montville).

The seed lot is subjected to a number of tests before it is determined suitable for the scaled-up fermentation stage, as detailed in safety and quality control. The seed lot should be pure and free of bacterial or fungal contamination, and fatty acid and sugar fermentation should be characterized. The seed lot must be highly toxigenic to produce maximum product yield. The absence of genes encoding for other toxin types should be confirmed (European Pharmacopoeia). Application of these steps ensures that only pure, highly toxigenic cultures are being used for industrial level production. This insures high toxin yields, and can prevent costly mistakes such as culturing a contaminated or mutated lot.

The fermenter is inoculated with cells from the growth lot. Industrial level fermentation must be done in the same conditions as the growth phase: anaerobic environment, same type of media, same pH and temperature. The process will use 50 L of fermentation media. Anaerobic conditions can be achieved with a 5 L/min flow of Nitrogen gas over the broth, and agitation at 50 rpm stimulates cell growth (Siegel). The fermenter will be chosen on a basis of cost and features, such as capacity, temperature, gas flow, sterilization, pH, and foaming control. Previous studies have shown that after 24 hours of incubation under similar condition, toxin production stopped at a maximum of 6.3×10^5 units/mL (Siegel). Thus, a 50L fermentation process should yield 3.15×10^{10} units BT-A.

e. Initial Precipitation

Upon completion of fermentation, the process of separating insoluble cells from the fermentation broth is the first step in product recovery. This step is crucial to the idealized process because it is the first step in decreasing sample volume. This makes for a more feasible batch size to work with efficiently, and reduces overall cost.

Neurotoxins from *Clostridium botulinum* are extracellular components, meaning they are contained within the fermentation solution, not within the cells. Cell disruption therefore is not performed, nor is filtration or centrifugation because the toxins are soluble components within the fermentation broth. The toxin is first precipitated out from the broth upon adjusting the pH to ~3.5 with treatment of a strong acid. Hydrochloric acid or Sulfuric acid are most commonly used. This pretreatment of the broth causes the toxins to aggregate into solids. The precipitate is left to settle at room temperature for up to 4 days.

The 50 L batch is fed into a large scale centrifuge where the aggregates are separated from the broth. A Rousselet Robatel Vertical Decanter Centrifuge model DRC 60 is used, with the properties listed below in Table 5. This centrifuge was chosen because it met capacity, and is suited to solid-liquid separations with high centrifugal force (G). Centrifugation is conducted at the max force of 2,267G for 1.1 hours. This time was calculated using the ideal centrifugation force of a protein aggregate, as listed in Table 3.3-1 in Lecture 5 BE 3340 notes. Calculation shown below:

$$G_t = 9 \times 10^6 \, sec$$
$$t_{min} = 9 \times 10^6 G_{max}$$
$$t_{min} = 3970 \, \text{sec} = 1.1 \, hr$$

The large volume of unwanted supernatant is removed (Duff et Al). A small sample size of a crystalline complex remains for further processing, all of which is performed batchwise at a lab-scale level. Centrifugation is used for separation throughout this process since Filtration is ineffective because of how Botulinum toxin adsorbs to filter mediums (Siegal)

	Bowl						Centrifugal Force (G)	Motor Power kW (HP)
Model	**Diameter mm (inches)**	**Height mm (inches)**	**Useful Volume liters (ft^3)**	**Maximum load kg (lb)**	**Sedimentation Capacity liters (ft^3)**	**Maximum Speed (RPM)**		
DRC 40	**400 (16)**	**225 (10)**	**18 (0.63)**	**22 (48)**	**7 (0.25)**	**3000**	**2010**	**2.2 (3)**
DRC 50	**500 (20)**	**320 (12.5)**	**35 (1.2)**	**43 (95)**	**18 (0.63)**	**2750**	**2113**	**4 (5)**
DRC 60	**600 (24)**	**350 (13.8)**	**60 (2.1)**	**75 (165)**	**27 (0.95)**	**2600**	**2267**	**9 (15)**

Table 5: Technical characteristics of Rousselet Robatel Vertical Decanter Centrifuges. With a batch size of 50 L, model DRC 60 is the centrifuge used. (*Technical Characteristics*)

The type A strain is proteolytic, a physiologic characteristic that cleaves the single chain precursor into the active dichain molecule made up of the heavy and light chain during fermentation (Simpson). Since the type A strain does this naturally, the crystalline structure obtained after this stage is fully activated, eliminating an activation step later in the process.

After the first centrifugation operation, many methods use distilled water for an initial precipitate cleansing. The sample is washed with appropriate amounts of distilled water for the sample size and the precipitate settles. The precipitate is re-collected by a standard lab-scale centrifuge at 2,000g for 10 minutes. This first precipitation and rinsing procedure yields approximately 80% recovery of the Botulinum toxin complex and is a 2.5-fold purification (Duff et al).

f. Isolation: Extraction

Extraction in general terms is the separation of a substance from a matrix, where the matrix can be solid or liquid. In the process of obtaining pure Botulinum toxin, solid-

liquid extraction is used. This process allows soluble components of a solid to be removed using an applicable solvent. In relation to BT-A, solvent extracts the desired toxic activity into a liquid form from the solid precipitate. Each extraction further purifies the level of purity in the toxin. To optimize this procedure to obtain the fastest and most complete extraction, the chosen solvent should have a large exchange surface and a quick diffusion rate with the particular solute ("Basic Knowledge: Solid-Liquid Extraction").

Method one: Resuspend the precipitate in 600 mL of 0.2 M sodium phosphate buffer, pH 6.0. Adjust the pH of dissolved toxin to 6.0 by sodium hydroxide (NaOH). Gently stir for 1 hour at 20°C. Centrifuge the extracted toxin at 2,500g for 10 minutes to clarify the separation. Save the extracted supernatant liquid, and repeat the process three times in 400 mL of 0.2 M sodium phosphate buffer. Then pool all four clarified extractions into one sample (Malizio et al).

Method Two: A second method for extraction uses 0.075 M Calcium Chloride (CaCl) as the extraction solvent, between pH 4-6.5. It was found that extraction of the desired toxic activity from the culture precipitate with minimum loss of impurity would be obtained through calcium chloride (Duff et al).

g. Purification of Toxin: Second Precipitation

A purified product is obtained through multiple precipitations. Ammonium sulfate *($(NH_4)_2SO_4$)* is the agent predominantly used in all procedures to precipitate the Botulinum toxin after treated with sodium phosphate buffer.

The ammonium sulfate is added to the extracted sample, bringing it to a 60% saturation for complete precipitation. The precipitate settles and is collected by centrifugation. It is then re-dissolved in 25 mL of 0.05 M sodium citrate buffer, pH 5.5 (Malizio et al). This prepares the precipitate as a liquid solution in order to use ion exchangers prior to column chromatographic steps.

h. Purification of BT-A Toxin Complex: Column Chromatography

Column Chromatography is a chemically based process used to purify individual chemical compounds from mixtures of compounds. There are a multitude of types of chromatography, differentiated by the chemical property it separates, and whether it utilizes gravity or added pressure. Spin chromatography columns are small, disposable, and can be packed with a variety of chromatographic media. They are sufficient but have a disadvantage of only using the force of gravity for flow. At a much higher level of efficiency is High Performance Liquid Chromatography (HPLC) columns, which utilize pumps to pass liquid through the column at a faster rate, and encompass a detector for quantitative results ("Gel Filtration Chromatography"). Some of the different types of HPLC include ion-exchange chromatography, affinity column chromatography, size-exclusion chromatography, and the Fast Protein Liquid Chromatography (FPLC) Mono-Q column. Each of these methods uses specific types of solid matrix to pack the column, often referred to as resin beads or sorbent particles made of silica or polymer. HPLC columns are made with smaller sorbent particles, ranging from 2-5 micrometers, which gives HPLC advantages in the higher resolution separations. It is certainty the more popular and widely accredited chromatographic technique. The solid matrix in the columns passes certain molecules, or selectivity binds to molecules, depending on the type of chromatography and unbound fractions wash through. If the bound molecules are the desired component, a

buffer solution is used to rinse the column thereafter to unbind those molecules and collect the eluted fraction.

Column Chromatography is extremely reliable in yielding pure desirable products. A series of three High Performance Liquid Chromatography columns are used to obtain a highly purified type A Botulinum toxin in the following process.

i. DEAE-Sephadex A-50 column

Sephadex is a widely used ion exchange media because of its high affinity, specificity, and easy technique of adsorbing proteins, peptides, and oligonucleotides on the basis of size ("Sephadex Ion Exchange Media"). It is a dry, micro sized, bead-formed gel with cross-linked dextran that swells upon adsorption.

DEAE-Sephadex (Diethylaminoethyl) is the first type of solid matrix most commonly used in purifying Botulinum toxin. It can either be added batchwise to the sodium citrate buffered solution, or can be loaded into a column and washed with the sodium citrate buffer. Both will perform the same function and obtain the same results. The solution is dialyzed for 4 h at room temperature with 3 dialysis changes at 1-h intervals, and centrifuged again to remove insolubles (Tepp). The extract is directly loaded into the DEAE-Sephadex A-50 column at a flow rate of 34-45 mL/hr (Malizio et al). The DEAE-Sephadex acts as filter aids and adsorbs the unwanted nucleic acids and a major protein band of ~38kDa. The unbound toxin complex washes through. The collected toxin fractions are then re-precipitated with ammonium sulfate, and collected by centrifugation. The solid precipitate is then redissolved in 0.05 M sodium phosphate buffer, pH 6.8. The toxin at this point is in its non-covalently bound complex with a hemagglutinating protein.

ii. CM-Sepharose chromatography column

Next, the solution is dialyzed at room temperature with 3 dialysis changes at 1-h intervals then loaded into a 0.9-cm by 12-cm CM-Sepharose chromatography column for further purification (Tepp). This column is composed of 6% agarose beads, with Carboxymethyl (CM) weak cation exchange groups, which selects for molecules of high molecular weight ("Gel Filtration Chromatography"). When washed with sodium phosphate buffer, unbound fractions containing the toxin are collected. These fractions contain approximately 95% pure Botulinum toxin (Tepp). The solution is precipitated with ammonium sulfate, and collected by centrifugation.

iii. Fast Protein Liquid Chromatography (FPLC) Mono-Q column

Lastly, the precipitate is re-suspended in sodium phosphate buffer, pH 8.0, and loaded into a Fast Protein Liquid Chromatography (FPLC) Mono-Q column. This process separates the toxin complex from the bound hemagglutinating protein to yield the purely isolated toxin. When loaded into the column, the resin binds the hemagglutinating protein with high affinity, which is bound to the toxin. The isolated toxin is then eluted from the column with 0.35 M NaCl, and the hemagglutinating protein remains bound to the resin.

i. Crystallization

The eluted fraction from the FPLC Mono Q-column is now essentially pure, and can therefore produce a pure toxin when crystallized. Ammonium sulfate solution is slowly

added to a final concentration of 0.9M while continuously stirring. Upon leaving the solution at 4°C for 1-4 weeks, crystals will form (Malizio et al).

j. Polishing: Preparation of Final Bulk Product

The purified toxin is suspended in a human serum medium. The specific toxicity of these final toxin containing solutions must be determined by a lethal dose (LD50) assay initially, and by a number of validated assays. For the LD50 assay, different amounts of toxin will be administered to Swiss Field Mice. The LD50 will be in the group with 50% lethality (Tepp et Al). Alternative assays can be used after an initial LD50 is used to calibrate them. Alternative assays include endopeptidase assay, phernic nerve diaphragm assay, and a mouse bioassay (with paralysis as endpoint) (Tepp et Al). The LD50 of the mice defines 1 unit of toxin (Ton et Al).

The final bulk product is transferred aseptically into sterile containers. Commercially available BOTOX® prepares 100 unit vials, consisting of 5ng toxin, 0.5mg human serum albumin, and 0.9mg NaCl. The vials are lyophilized for transport and storage, and resuspended in 0.9% saline solution for injection (Ton et Al). They are often vacuum-dried or freeze-dried and kept at -20°C, which has found to effectively maintain the stability and level of toxic activity without any appreciable loss of the sample vial (Malizio et al). Other commercially available products use compounds like lactose and sodium succinate; and are available in powder or solution form (Ton et Al). Our product will mimic the composition of BOTOX® since this is determined most effective for clinical use. Labeling of the individual containers requires the units of toxin per vial and the name and volume of diluent added (European Pharmacopedia). If fermentation produces $3.15x10^{10}$ mouse LD50 toxin ($3.15x10^{10}$ units) total, and purification results in minimal loses, then this process has the potential to produce $3.15x10^{8}$ vials.

III. Safety and Quality Control

The nature of this process calls for extreme care for worker and public safety because bacteria with the ability to produce a potentially lethal toxin are being cultured in large numbers. If safety protocol is breach, there is potential for an epidemic outbreak. *C. botulinum* is classified as a Biosafety Level-2 substance. Protocols are outlined in the Center for Disease Control's publication ("Biosafety in Microbial and Biomedical Laboratories"). Additionally, a number of quality control steps will be applied at several points in the production process to prevent against contamination and insure production of a pure toxin.

a. Before Inoculation of Fermenter

Before inoculating the seed lot into the fermenter, cultures from the initial growth phase must be tested. Presence of pure *C. botulinum* is confirmed by colony and cell morphology. Fatty acid, sugar fermentation, and proteolytic profiles are determined. The presence of the gene for type A neurotoxin must be confirmed, as well as the absence of any other type. An acute toxicity assay is applied to determine the toxicity of the strain (European Pharmacopedia). A seed lot from the growth phase that falls within acceptable limits for each of these assays can be inoculated into the fermenter for scaled-up toxin

production. This insures that only pure, highly toxic strains are used for industrial level processing.

b. Before Preparation of Final Bulk Product

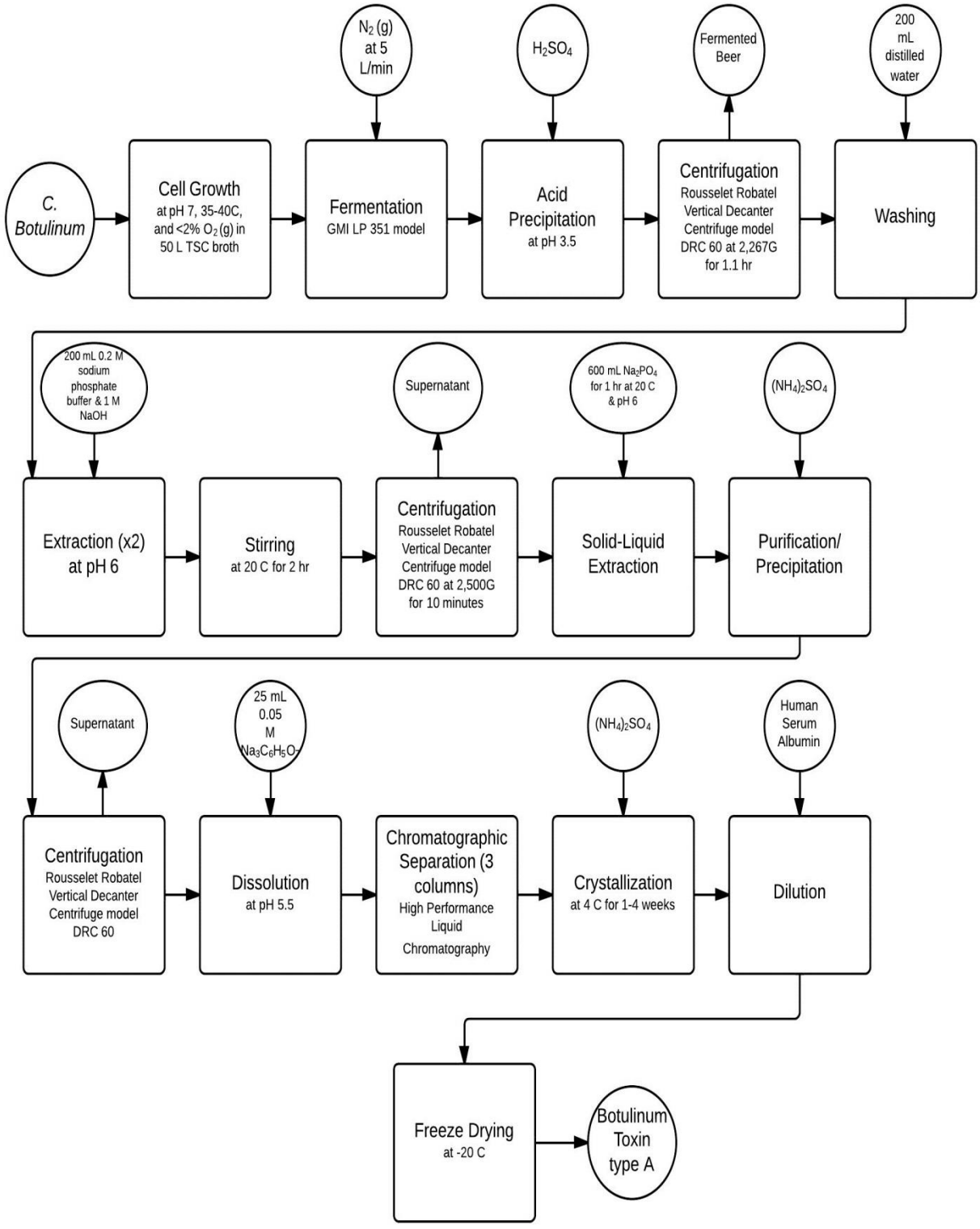
The purified toxin is tested before preparing the final bulk product. Removal of nucleic acids and residual reagents from purification are confirmed by suitable limit tests. Presence of the specific Botulinum Type A neurotoxin is confirmed by an immunochemical test. A lethal dose assay is applied to determine the specific activity of the batch in units of LD50/mg-protein. Protein concentration is determined by a suitable assay. Protein composition is determined by electrophoresis or size-exclusion chromatography (European Pharmacopedia). The purified toxin may only be used for preparation of the final bulk product if it falls within acceptable limits for each of these assays.

IV. Chosen Methods

a. Introduction

The methods for this process were strategically selected in order to reach the goal of producing 100 unit vials of Botulinum toxin A from a 50 L batch. The chosen process steps address the group's primary design concerns including cost efficiency, safety regulations, and environmental safety issues. Along with these limitations in mind, the steps were mathematically analyzed to determine which option would produce a greater yield. Optimal growth conditions were coupled with high quality raw materials in order to ensure a maximum collection of the raw toxin. The chosen equipment operates within the necessary settings and can adequately handle the amount of product involved in each step. Through idealizing the materials and equipment, the goal is to reduce the error percentage throughout the process. Considering this process deals with a very small initial concentration of toxin, accuracy is extremely important in producing the highest possible yield of Botulinum toxin A.

b. Finalized Flowchart



c. Growth and Fermentation

The seed lot for fermentation will be a sample of *C. botulinum* spores which will germinate when placed in conditions optimal for its growth. Spores are more stable and less active, making transportation and storage much easier than living cells.

Tryptose Sulfite Cycloserine (TSC) broth has been selected as the 50 L growth medium. This medium proved to be the most practical choice because it is selective for *Clostridia* strains. The differential aspect of the broth will help reduce the possibility of contaminants during the growth stage. With less competition, the *C. botulinum* strains can more easily reach the maximum doubling time of 30 minutes, and will help improve the process yield. This medium is generally the safest of the three growth media options, considering only two of its components are considered hazardous. Sodium metabisulfate is an irritant and ferric ammonium citrate is believed to be harmful when introduced into water systems. However, these risks can easily be monitored by following proper safety and waste disposal regulations.

The fermenter used will be the LP 351 model from GMI. The fermenter was selected for its 50 L capacity, gas flow, pH, temperature control, and agitation ability. Its modular design makes it versatile for other biotech applications. Fermentation will be carried out over a 24 hour period at 37°C, pH of 7, 2% oxygen maintained with a flow of Nitrogen gas over the broth at 5 L/min, and agitation at 50 rpm. These conditions will encourage optimal cell growth and toxin production.

Based on previous studies, fermentation is expected to yield a broth with toxin concentration of 6.3×10^5 units/mL (Siegel). Based on an LD50 of 1.2 ng for a mouse, this converts to 7.56×10^{-4} g/mL. The low concentration of solute justifies the large capacity of the fermentation process, as purification processes may lose efficiency at low concentrations.

d. Initial Precipitation

Upon completion of fermentation, the process of separating insoluble cells from the fermentation broth is the first step in product recovery. This step is crucial to the idealized process because it is the first step in decreasing sample volume. This makes for a more feasible batch size to work with efficiently, and reduces overall cost.

Neurotoxins from *Clostridium botulinum* are extracellular components, meaning they are contained within the fermentation solution, not within the cells. Cell disruption therefore is not performed, nor is filtration or centrifugation at this stage because the toxins are soluble components within the fermentation broth. The toxin is first precipitated out from the broth upon adjusting the pH to 3.5 with treatment of 3 N Sulfuric Acid. This pretreatment of the broth causes the toxins to aggregate into solids. Knowing that the concentration of BT-A is 1.2 ng/LD50 as stated in the fermentation process, approximately **37.8 g** will initially precipitate out from the broth.

$$3.15 \times 10^{10} \text{ LD50} \times (1.2 \text{ ng/LD50}) \times \left(\frac{1\text{g}}{1 \times 10^9 \text{ng}}\right) = \mathbf{37.8\ g}$$

The precipitate is left to settle at room temperature for 1 hour (Tepp). The 50 L batch is fed into a large scale centrifuge where the aggregates are separated from the broth. A Rousselet Robatel Vertical Decanter Centrifuge model DRC 60 will be used, with the

properties listed below in Table 5. This centrifuge was chosen because it met capacity, and is suited to solid-liquid separations with high centrifugal force (G). Centrifugation is conducted at the max force of 2,267G for 1.1 hours. This time was calculated using the ideal centrifugation force of a protein aggregate (G_t=9x10^6 sec) as listed in Table 3.3-1 in Lecture 5 BE 3340 notes. Calculation shown below:

$$G_t = 9 \times 10^6 \text{ sec}$$
$$t_{min} = 9 \times 10^6 G_{max}$$
$$t_{min} = 3970 \text{ sec} = 1.1\ hr$$

The 37.8 grams of precipitated toxin complex will accumulate after centrifugation, and the large volume of unwanted supernatant is removed (Duff et al). Centrifugation is the chosen method of separation throughout this process because Botulinum toxin adsorbs to filter mediums, making filtration ineffective (Siegal).

The type A strain is proteolytic, a physiologic characteristic that cleaves the single chain precursor into the active dichain molecule, made up of the heavy and light chain (Simpson). Since the type A strain does this naturally during fermentation, the crystalline structure obtained after this stage is fully activated. After collecting precipitate from the first centrifugation operation, 200 mL of distilled water is applied for an initial cleansing.

e. Isolation: Extraction

Extraction in general terms is the mixing of two immiscible phases to transfer a solute from one phase to another (BE 3340 Lecture 8 notes). In the process of obtaining pure Botulinum toxin, liquid-liquid extraction is used, and more specifically, repeated or staged extractions. This process allows the desired toxic activity in the precipitate-water solution to be extracted out using sodium phosphate buffer. Each extraction further isolates the toxic activity in the toxin solution. Method one was chosen because sodium phosphate buffer is the agent used in almost all published procedures of Botulinum toxin type A.

According to the extraction procedure of William H. Tepp, the Botulinum toxin precipitate suspended in the distilled water will be extracted twice with 200 mL of 0.2 M sodium phosphate buffer, at pH 6.0. The pH of 6 is obtained by adding small amounts of 1 M sodium hydroxide (NaOH). It is gently stirred for 2 hours at room temperature, and then centrifuged at 2,500g for 10 minutes to clarify the separation. The supernatant with the desired toxic activity is collected. The two extractions yield approximately 80% recovery of the Botulinum toxin complex and is a 2.5-fold purification (Duff et al). Therefore, the concentration of the amount extracted is 151.2 mg/mL.

$$\frac{37.8\ g}{200\ mL} = 189\ {}^{mg}/_{mL}$$

$$189\ {}^{mg}/_{mL}\ x\ 0.80 = 151.2\ {}^{mg}/_{mL}$$

f. Purification of Toxin: Second Precipitation

After impurities are extracted, a further purified product is obtained through precipitation the complex out again. Solid ammonium sulfate *(*39g/mL*)* is the agent used to

precipitate the Botulinum toxin after treated with sodium phosphate buffer. The ammonium sulfate is added to the extracted pool. The precipitate settles and is collected by centrifugation.

It is then re-dissolved in 1 mL of 0.05 M sodium citrate buffer, pH 5.5 (Tepp). This prepares the precipitate as a liquid solution in order to use ion exchangers prior to column chromatographic steps.

g. Purification of BT-A Toxin Complex: Column Chromatography

Column Chromatography is a very selective chemically based process used to purify individual chemical compounds from mixtures. There are a multitude of types of chromatography, differentiated by the chemical property it separates, and whether it utilizes gravity or added pressure. The chosen type for this study is High Performance Liquid Chromatography (HPLC) columns because it utilizes pumps to pass liquid through the column at a faster rate, and encompass a detector for quantitative results ("Gel Filtration Chromatography"). The different types of HPLC columns used are ion-exchange and Fast Protein Liquid Chromatography (FPLC). Each of these methods uses specific types of solid matrix to pack the column, often referred to as resin beads or sorbent particles made of silica or polymer. HPLC columns are made with smaller sorbent particles, ranging from 2-5 μm, which gives HPLC advantages in the higher resolution separations. Column Chromatography is extremely reliable in yielding pure desirable products. A series of three High Performance Liquid Chromatography columns are used to obtain a highly purified type A Botulinum toxin in the following process.

i. DEAE-Sephadex A-50 column

Sephadex is a widely used ion exchange media because of its high affinity, specificity, and easy technique of adsorbing proteins, peptides, and oligonucleotides on the basis of size ("Sephadex Ion Exchange Media"). It is a dry, micro sized, bead-formed gel with cross-linked dextran that swells upon adsorption.

DEAE-Sephadex (Diethylaminoethyl) is the first type of solid matrix most commonly used in purifying Botulinum toxin. First, the supernatant obtained from extraction is dialyzed for 4 h at room temperature with 3 dialysis changes at 1-h intervals, and centrifuged again to remove insolubles (Tepp). The extract is then directly loaded into the DEAE-Sephadex A-50 column at a flow rate of 34-45 mL/hr (Malizio et al). The DEAE-Sephadex acts as filter aids and adsorbs the unwanted nucleic acids and a major protein band of ~38kDa. The unbound toxin complex washes through. The collected toxin fractions are then re-precipitated with ammonium sulfate (39g/ml), and collected by centrifugation. The solid precipitate is then redissolved in 0.05 M sodium citrate buffer, pH 6.0 (Tepp). The toxin at this point is in its non-covalently bound complex with a hemagglutinating protein.

ii. CM-Sepharose chromatography column

Next, the solution is dialyzed at room temperature with 3 dialysis changes at 1-h intervals then loaded into a 0.9-cm by 12-cm CM-Sepharose chromatography column for further purification (Tepp). This column is composed of 6% agarose beads, with Carboxymethyl (CM) weak cation exchange groups, which selects for molecules of high molecular weight ("Gel Filtration Chromatography"). When washed with sodium

phosphate buffer, unbound fractions containing the toxin are collected. These fractions contain approximately 95% pure botulinum toxin (Tepp). The solution is precipitated with ammonium sulfate, and collected by centrifugation.

iii. Fast Protein Liquid Chromatography (FPLC) Mono-Q column

Lastly, the precipitate is re-suspended in sodium phosphate buffer, pH 8.0, and loaded into a Fast Protein Liquid Chromatography (FPLC) Mono-Q column. This process separates the toxin complex from the bound hemagglutinating protein to yield the purely isolated toxin. When loaded into the column, the resin binds the hemagglutinating protein with high affinity, which is bound to the toxin. The isolated toxin is then eluted from the column with 0.35 M NaCl, and the hemagglutinating protein remains bound to the resin.

h. Crystallization

The eluted fraction from the FPLC Mono Q-column is now essentially pure, and can therefore produce a pure toxin when crystallized. Ammonium sulfate solution is slowly added to a final concentration of 0.9M while continuously stirring. Upon leaving the solution at 4°C for 1-4 weeks, crystals will form (Malizio et al).

i. Preparation of Final Product

Crystallized BT-A is diluted with human serum albumin. Because the dilution will be separated into 2.5 mL vials, and each is to contain 100 units BT-A, a final concentration of 40 units/mL is desired. Assuming that 30.24g (25,200,000,000 units) BT-A is recovered from the purification processes, 630,208 L of human serum albumin must be added to dilute the sample to the desired toxin concentration.

The diluted medium will be distributed into separate 2.5 mL vials, each containing 100 units BT-A. The solution in the vials will be vacuum freeze dried for storage and transportation, to be resuspended with 2.5 mL of 4% saline solution by a trained healthcare provider for injection. The toxin is more stable and less susceptible to hydrolysis in the dried state.

The 630,208 L of solution is to be divided into 2.5 mL vials, yielding 252,083,000 vials total. It should be noted that the freeze dried toxin can be stored for years when lyophilized and frozen ("Thermo Scientific Tech Tip #43"). Thus some of the crystallized toxin can be diluted, separated, and prepared, while the rest can be stored in bulk and prepared when needed.

V. Budget

Component	Amount	Pricing	Quantity	Cost
Tryptose	833.33 g	500 g @ $92.10	2	$184.20
Yeast extract	277.78 g	500 g @ $72.50	1	$72.50
Soytone	277.78 g	500 g @ $96.35	1	$96.35
Ferric ammonium citrate	55.56 g	100 g @ $46.20	1	$46.20
Sodium metabisulfite	55.56 g	100 g @ $22.60	1	$22.60
Distilled water	50.2 liter	1 gal @ $0.98	14	$13.72
N_2 gas	7,200 liter	7,200 liter @ $2066.90	1	$2066.90
3 N H_2SO_4	1 liter	1 liter @ $29.70	1	$29.70
0.2 M Na_2PO_4	200 mL	50 tabs @ $57.10	1	$57.10
1 M NaOH	Small amt	50 mL @ $21.30	1	$21.30
$(NH_4)_2SO_4$		25 tons @ $175.00	1	$175.00
$Na_3C_6H_5O$	1 mL	250 mL @ $30.00	1	$30.00
Human Serum Albumin	630,208 liter	250 mL @ $3,385.00	251,994	$853,000,000
DEAE-Sephadex resin	50 g	50 g @ $282.00	1	$282.00
CM Sepharose	50 mL	50 mL @ $99.00	1	$99.00
Mono Q 5/50 GL column	5cm x 5mm	1 column @ $1,760.00	1	$1,760.00
		Total		**$853,004,963.80**

VI. Conclusion

Although the process is complex, the outlook for BT-A use in patients is expanding greatly. With more research, BT-A is branching away from cosmetic applications and is becoming a prominent part of a dynamic treatment for cerebral palsy. Through this design process, the objective of producing vials of toxin each containing 100 units can be reached in a safe, and time effective method. Each vial can be processed into different dosages in order to help a number of different patients. If conditions are optimized and little to no error occurs during the process, then up to 315,000,000 vials containing 100 units can be produced in one batch. With the overall budget, this averages $2.71 per vial; so although the budget seems large, the cost per vial is fairly low. Each of these vials has the potential to greatly improve the life quality of a patient with cerebral palsy.

VII. References

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